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THE PHYSIOLOGICAL BASES FOR MICROBIAL BAROTOLERANCE.(U)
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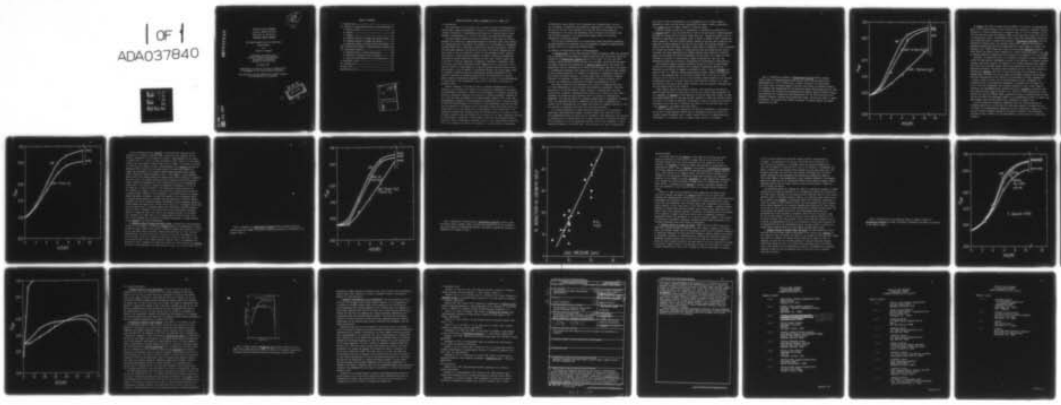

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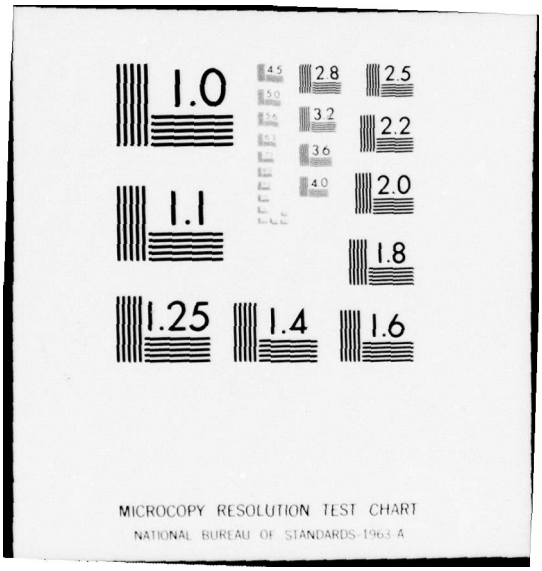
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TECHNICAL REPORT NUMBER 5

The Physiological Bases for Microbial Barotolerance

by

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31 March 1977

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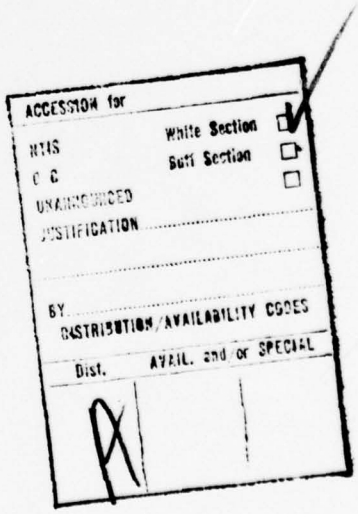
TABLE OF CONTENTS



REPORT FOR PERIOD FROM 31 DECEMBER 1975 TO 1 APRIL 1977

I. Introduction

The stated goals of the project are to define the bases for differences in barotolerance among microorganisms in terms of the pressure sensitivities of specific biochemical or physiological processes and to explore ways of using pressure advantageously as a tool for both basic and applied microbiology.

The past support period has seen the extension of these goals to include studies of the effects of high-pressure gases on microbial cells. These studies are complicated ones technically, and moreover, it is necessary to distinguish between effects due to hydrostatic pressure and specific gas effects. However, the work seems important and pertinent in view of the recent finding that man may be able to work at pressures as great as 150 atmospheres {atm}. Survival and function at these extreme pressures is thought to depend on antagonistic interactions between hydrostatic pressure and the inert, narcotic gases used as ballast to keep the lungs from collapsing in hyperbaric atmospheres. Some of our past work {Fenn and Marquis, 1968} suggested that at least part of the narcotic effects of inert gases and of N_2O are general cellular effects, and it was possible to slow the growth of unicellular bacteria with these gases. There is currently a critical need for biochemically detailed information on the actions of narcotic or anesthetic gases and on the interactions of narcotic gases, oxygen and hydrostatic pressure. We hope to be able to use microorganisms to obtain some of this information, and a part of our work in this area is described below.

Another component of the project that is currently demanding more of our attention is the study of the effects of relatively low pressures on the growth and physiology of bacteria. A review of the development of microbial barobiology reveals an inordinate interest in the effects of extreme biospheric pressures of up to 1100 atm that are ambient in the abyssal-hadal regions of the ocean. This understandable tendency to focus attention on extremes has led to relative neglect by microbial barobiologists of the effects of lower pressures, less than 200 atm, that are ambient in the regions of the continental shelves and slopes and in the upper levels of the open ocean. Part of this neglect can be traced also to the laboratory findings that pressures below 200 atm have little or no effect on most bacteria. However, these nul findings can be related to the usual laboratory procedure of setting growth conditions to near optimal values and then applying pressure. Growth in natural environments ordinarily does not occur under optimal conditions, and if one considers the effects of pressure on bacterial growth in nonoptimal

circumstances, major effects of low pressures can be demonstrated. In fact, it is possible to completely stop growth by application of pressures less than 100 atm. We now feel that pressures less than 200 atm are more important ecologically in the ocean, and presumably also in bodies of fresh water, than are the high pressures in the abyssal-hadal regions where the microbial population is relatively sparse.

We have also continued work on the physiologic bases from growth inhibition by high pressure and on specific uses for hydrostatic pressure in applied microbiology. Some of our results are described below.

II. Effects of High-pressure Gases on Microorganisms.

A. Nitrous oxide. We had found previously {Fenn and Marquis, 1968} that bacteria can be inhibited in their growth by narcotic gases and that the potency series for inhibition of Streptococcus faecalis growth is Xe or N_2O > Ar > N_2 > He. More recently, Enfors and Molin {1975} reported that nitrogen, nitrous oxide and argon are highly inhibitory for germination of Bacillus cereus spores. The process of germination could be inhibited by as little as 50 atm argon, and 64% of the spores in a germinating population were stopped by only one atm of nitrous oxide. Because of this extreme sensitivity, it seemed that inhibition of germination might be a more readily monitored response to narcotic gases than is inhibition of growth. Therefore, we undertook to repeat the observations of Enfors and Molin and to evaluate the comparative sensitivities of growth and germination to N_2O.

Our results indicated that germination can, indeed, be inhibited with N_2O. For these experiments, we used L-alanine and adenosine as germinants. They were dissolved in a 61.3% {wt/volume} aqueous solution of Carbowax 4000, and this solution plus a magnetic stirring bar were placed at the bottom of a tube. Then a suspension of spores was layer carefully over the solution of Carbowax plus germinants. The tube was placed in a pressure chamber made of nonmagnetic, stainless steel, and compressed N_2O from a commercial tank was used to pressurize the chamber. After pressurization, the chamber was placed on a magnetic stirrer so that the solution of germinants was mixed with the spore suspension.

In a typical experiment, a spore population exposed only to air underwent essentially 100% germination during a five-hour period with about 30% of the cells germinating within the first hour of incubation. Here germination was assessed by observing loss of refractility with the phase microscope. The spores were fixed with 5% formalin to stop the germination process. In the same experiment, a spore population exposed to 10 atm N_2O plus 1 atm air showed only about 7% germination

even after 23 hours. Decompression of the suspension led to nearly complete germination within one hour unless formaldehyde was added. Clearly, germination of B. cereus spores is highly sensitive to narcotic gases.

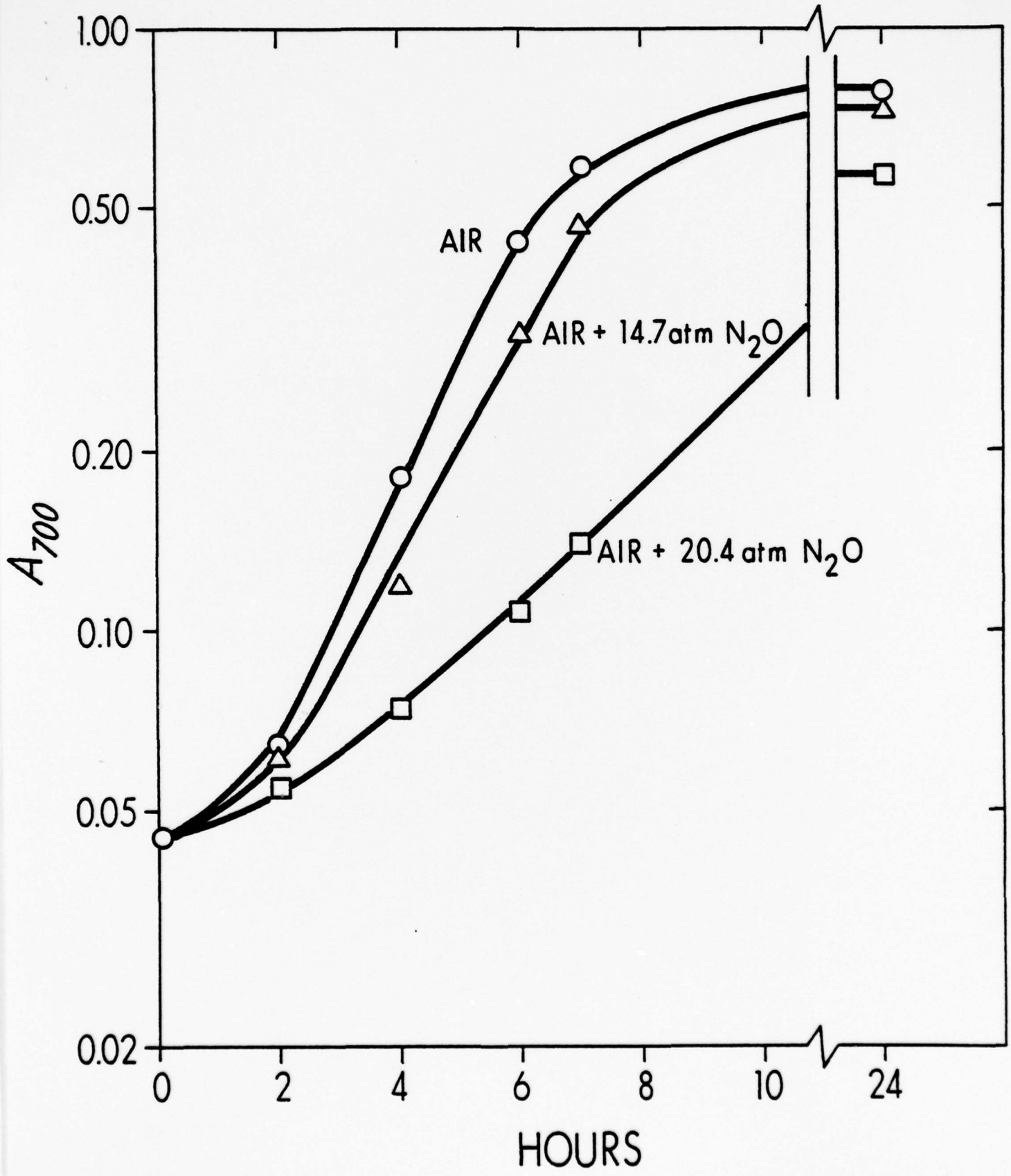
However, during the same set of experiments, we assessed the sensitivity of S. feacalis growth to N_2O and found that 10 atm of the gas caused a readily apparent decrease in average size of colonies formed from cell suspensions spread over the surfaces of agar plates. For example, after 24 hours of growth at 24°C, colonies on plates exposed to air were clearly visible, whereas no colonies were visible to the naked eye on plates exposed to air plus 10 atm of N_2O. The bacteria exposed to nitrous oxide were not killed, and after several days of incubation, a full complement of colonies was visible. Growth in liquid culture also was slowed by N_2O, and we concluded that spore germination was perhaps somewhat more sensitive to narcotic gases than was growth but that the difference was not major. As a result of these findings, we decided to continue work on growth responses but feel that the germination system may be useful in deciphering the biochemistry of gas narcosis.

More detailed studies on growth inhibition by N_2O for cells in liquid media showed that the response included both a slowing of exponential growth and a reduction in cell yield. An example is presented in Fig. 1. Here S. faecalis was grown at 22°C in a complex, tryptone-glucose-yeast-extract medium. The generation time, or mass doubling time, in the culture exposed only to air was 1.5 hours. Addition of 14.7 atm N_2O to the air in the pressure chamber resulted in an increase in generation time to 1.6 hours, while 20.4 atm caused an increase to 3.2 hours. The effects of the gas on final culture densities are also apparent, although they are less marked than the rate effects.

These results confirm our earlier conclusion that bacteria are affected by narcotic gases and that at least part of the action of these gases is general for all organisms. S. faecalis is less sensitive to nitrous oxide than is man, for whom the anesthetizing dose is less than one atm. However, it seems that bacteria should be useful in the study of anesthetic mechanisms in terms of molecular and cellular responses, and we plan now to determine just what it is that nitrous oxide does to S. faecalis to slow growth and reduce yield.

A major component of the progress in the reported experiments is technologic. We now have a large pressure chamber made of nonmagnetic steel that allows for accurate growth assessments with a single stirred culture in a high-pressure gas environment. It is possible also to incubate agar plates in the chamber.

Fig. 1. Inhibition of growth of Streptococcus faecalis 9790 by nitrous oxide. Flasks of tryptone-glucose-yeast-extract broth were inoculated with approximately 2.5×10^7 cells/ml from a 24-hour culture in the same medium. The flasks, which contained magnetic stirrers, were placed in pressure chambers. The chambers were pressurized with the indicated gases. Medical-grade N_2O was used. At intervals, the cultures were decompressed and sampled. Optical densities $\{A_{700}\}$ were measured with a Beckman DU spectrophotometer set for 700 nm and with 1-cm lightpath cuvettes. Cultures were diluted with water, and care was taken to remove gas bubbles. The cultures were stirred throughout the growth period. The growth temperature was 22°C.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A_{700}
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AIR + 20.4 atm N_2O
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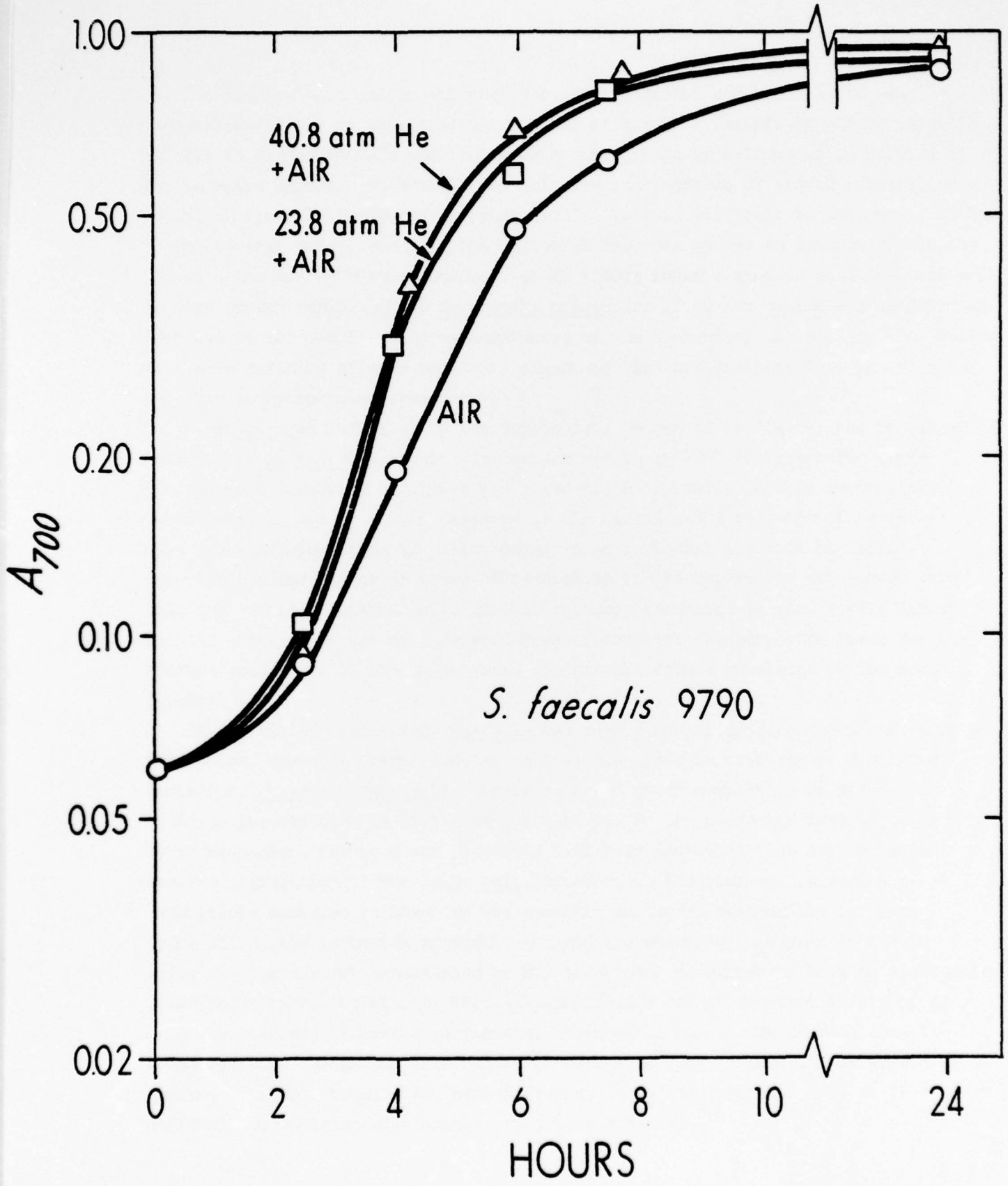
HOURS

B. Helium. The study of Brauer and Way {1970} of the effects of high-pressure gases on the righting reflex of mice indicated that helium has a negative narcotic potential of -0.045, compared with a positive value of 1.00 for nitrogen. In past work with bacteria {Fenn and Marquis, 1968} we had found that helium had little or no antibacterial effect, and Macdonald {1975} found that high-pressure helium had less inhibitory effect on growth and division of Tetrahymena pyriformis than did hydrostatic pressure alone. In contrast, Kenis {1971} reported that helium at pressures greater than 100 atm was inhibitory for a wide range of bacteria, and Buchheit et al. {1966} found that a pHe of 60 atm slowed Neurospora growth to about 67% of the control value. Schlamm et al. {1974} found that growth of E. coli in minimal medium was enhanced by 68 atm helium in the presence of 0.2 atm oxygen.

Certainly, if helium has little or no narcotic potential, it would be extremely useful for many types of experiments in which it is desirable to transmit pressure through a gas phase. However, the results of the studies mentioned above suggest that there may be specific helium effects that differ from effects due solely to hydrostatic pressure. The growth curves presented in Fig. 2 show that helium can stimulate S. faecalis. Here, 23.8 or 40.8 atm helium, in the presence of air, significantly increased the rate of growth and slightly enhanced culture yields. The mass doubling time of the culture in air was about 1.6 hours compared with a value of about 1.0 hours for the helium-treated cultures. Strangely, the stimulatory effect was relatively independent of pressure over the range from 23.8 to 40.8 atm. Experiments are currently underway to test a wider range of pressures. The growth stimulation reported here for S. faecalis is different than that reported for E. coli by Schlamm et al. {1974}. In their experiments, helium reduced the lag before growth began, apparently because of enhanced uptake of iron, but did not affect the subsequent growth. In our experiments, effects on growth lag were minimal, and the major effect was a stimulation of growth rate.

We also carried out companion experiments with the cultures enclosed in stoppered glass vials. In this way, the cells were exposed to pressure but not to helium. At the experimental temperature of 22°C, hydrostatic pressures of 23.8 and 40.8 atm were slightly inhibitory for growth. Therefore, the stimulation observed in Fig. 2 seems to be due specifically to helium and not to hydrostatic pressure.

Fig. 2. Stimulation of streptococcal growth by helium. The general experimental procedure is described in the legend to Fig. 1.


1.00
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A_{700}
40.8 atm He
+AIR
23.8 atm He
+AIR
AIR
S. faecalis 9790
0
2
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6
8
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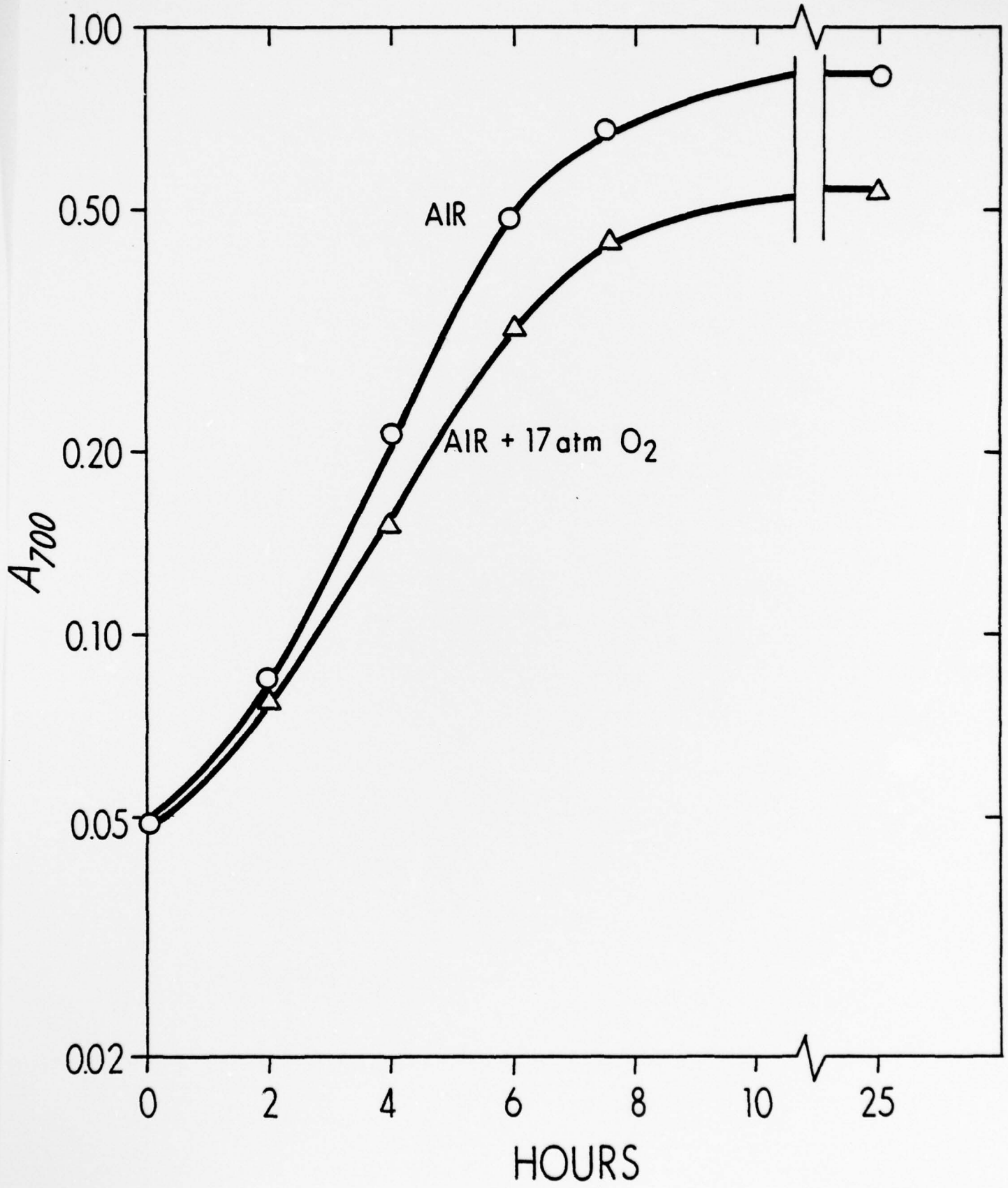
24
HOURS

The finding that helium stimulates bacterial growth and replication was unexpected. It may be that growth responses of bacteria faithfully mirror narcotic effects in higher animals and that growth stimulation by helium can be related to its negative narcotic potential. The relative independence of growth stimulation on pHe in the relatively narrow range studied here is difficult to interpret, although it may be that some element in the bacterial response system is saturated with the gas at a low concentration. Buchheit et al {1966} found a similar puzzling dose effect in that growth inhibition of Neurospora crassa due to 60 atm helium was as great as that due to 120 atm of the gas. Regardless of the mechanism, it is clear that helium does have definite effects on living organisms that are distinct from the effects due only to hydrostatic pressure.

C. Oxygen. As indicated by the sample data presented in Fig. 3, the responses of cultures of S. faecalis growing in complex medium at 22°C to hyperbaric oxygen include both a slowing of growth and a reduction in yield. In this particular experiment, 17 atm O_2 in the presence of air caused a 36% reduction in growth rate and a 35% reduction in yield. Oxygen also is lethal for this bacterium, and viable counts after 25 hours of incubation in the hyperbaric atmosphere were some 20% of those obtained with similar cultures incubated in air. Total, direct counts, obtained by use of a Petroff-Hausser counting chamber, were lower for the culture exposed to 17 atm O_2 but were predictable from a knowledge of the optical density.

Oxygen killed cells of S. faecalis had nearly normal appearance in the phase microscope. However, oxygen induced large-scale lysis in cultures of a related bacterium, Streptococcus lactis. Lysis occurred after completion of growth and it could be induced with as little as 3 atm of O_2. We are currently investigating the lytic mechanism. Previous work reported last year indicated that oxygen caused massive degradation of RNA in E. coli. However, in the course of a series of experiments designed to identify the specific nucleases responsible for this degradation, the bacterium gradually changed its response to oxygen to one in which killing was not accompanied by RNA breakdown. We currently have no reasonable interpretation of the change. When a lyophilized stock of the test strain of E. coli was used as a source of organisms, there still was no RNA degradation. It seems that the change may have had to do with some minor change in the growth medium. Although, because the medium used was a defined, minimal one, it is difficult to envision what change could have occurred.

Fig. 3. Inhibition of growth of Streptococcus faecalis 9790 by oxygen. The general experimental procedure is described in the legend to Fig. 1.


1.00
0.50
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A_{700}
AIR
AIR + 17 atm O_2
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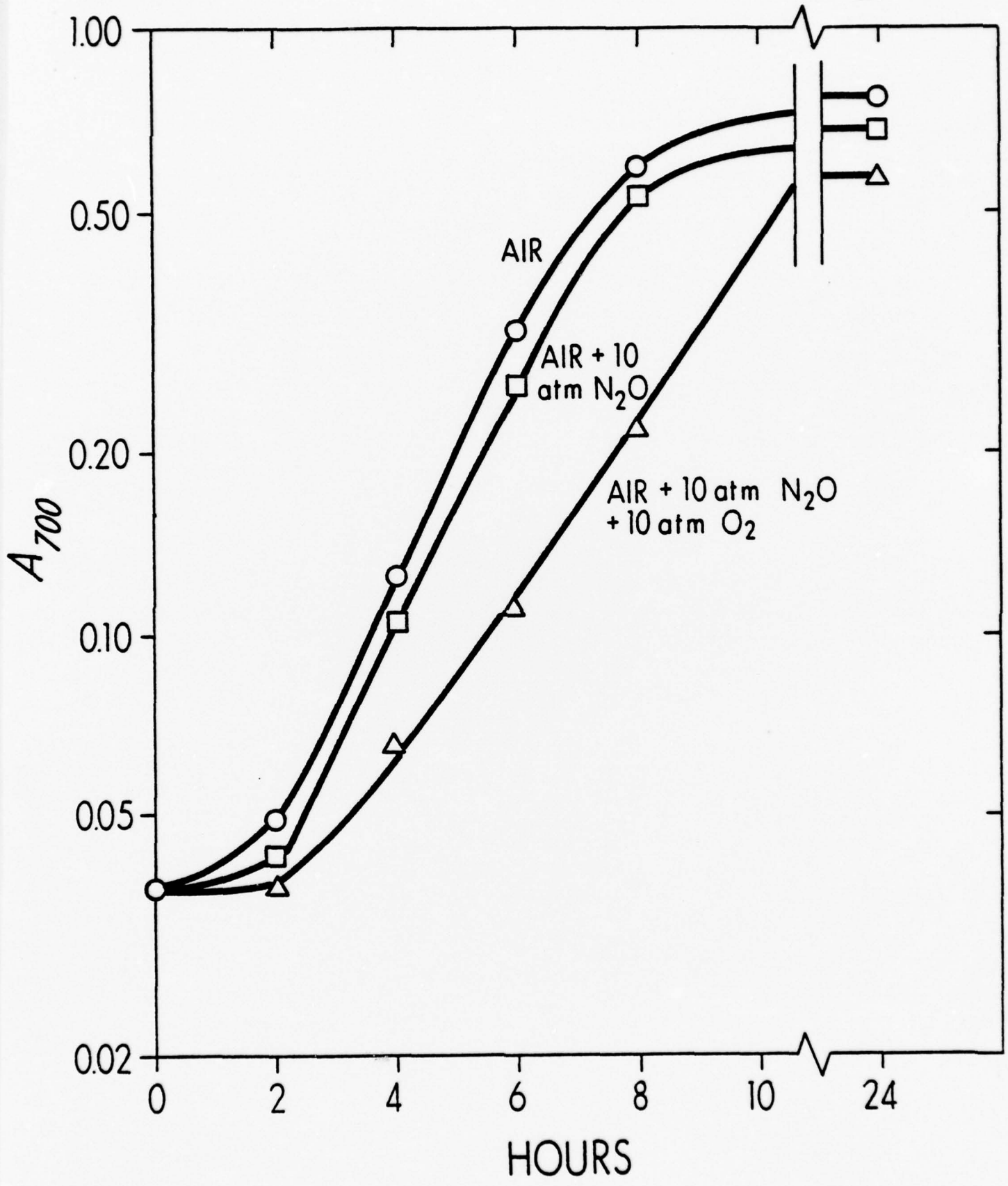
HOURS

In our experiments with S. faecalis, we were somewhat perplexed by what appeared to be a greatly increased oxygen tolerance of the organism. In our previous experiments, it was possible to achieve a 50% inhibition of the extent of growth with 3 to 4 atm of O_2 {Fenn and Marquis, 1968}. However, in the present series, this same response required some 25 atm of oxygen. In an effort to discern the basis for this change, we used a defined medium for S. faecalis described by Matsumura {1975}. Growth in this medium proved to be much more sensitive to oxygen than was growth in the complex, tryptone-glucose-yeast-extract medium used to obtain the results presented in Fig. 3. For example, in one experiment growth yield in the defined medium was reduced nearly 70% in response to treatment with 10.2 atm O_2. The defined medium was made up initially with 50 mM phosphate buffer. Because of precipitation that occurred when the pH of the medium was raised above 7, we decided to reduce the phosphate concentration to 25 mM. To our surprise, we found that growth in the medium with reduced phosphate was less sensitive to oxygen. In fact, it was only about as sensitive as was growth in complex medium. Addition of more phosphate to the medium resulted in enhanced oxygen sensitivity. Moreover, we found that addition of 0.1 M phosphate buffer to complex medium resulted in markedly increased sensitivity to both the growth-inhibitory and lethal actions of oxygen. For example, in one experiment, growth in complex medium was reduced 34% in extent by 20.4 atm of O_2. A companion culture with the same medium plus 0.1 M phosphate buffer was inhibited by some 66%. Moreover, after 24 hours of incubation, the viable count in the medium without phosphate exposed to oxygen was 24% of the count in the unexposed culture. In the medium with phosphate, the viable count in the oxygen-exposed culture was only 11% of that in the unexposed one. The basis for phosphate enhancement of oxygen toxicity for S. faecalis is now being investigated.

D. Combined actions of oxygen and nitrous oxide. As shown by the data presented in Fig. 4, it is possible to inhibit S. faecalis growth with combinations of oxygen and nitrous oxide. Again, there is both a slowing of growth and a reduction in yield. We had previously found that narcotic gases could potentiate the toxic action of oxygen.However, the effect seen here is little, if any, more than additive. Ten atm of N_2O plus 10 atm of O_2 produced a reduction in yield of about 39%, or about what one would expect from a simple additive combined action. The aggregate data presented in Fig. 5 suggest that oxygen acts simply as a narcotic gas for S. faecalis and that it has approximately the same potential as N_2O, as one would expect from its

Fig. 4. Inhibition of Streptococcus faecalis growth by nitrous oxide and nitrous oxide plus oxygen. The general experimental procedure is described in the legend to Fig. 1.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AIR + 10 atm N_2O
AIR + 10 atm N_2O + 10 atm O_2
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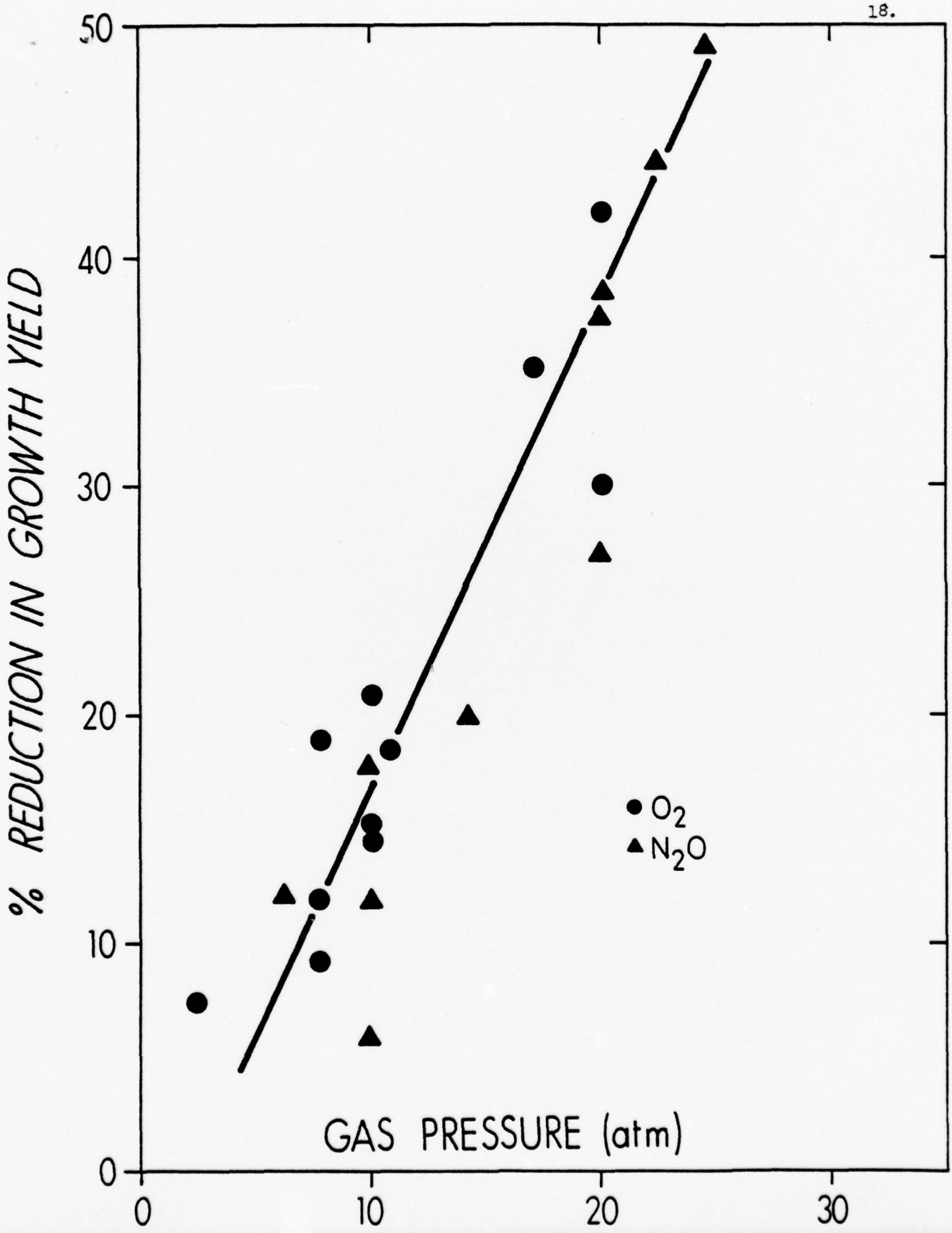
HOURS

Fig. 5. Reductions in growth yield of Streptococcus faecalis cultures caused by nitrous oxide or oxygen. Control cultures were incubated in an air atmosphere, and air was present in pressurized cultures. The general experimental procedure is described in the legend to Fig. 1.


% REDUCTION IN GROWTH YIELD
GAS PRESSURE (atm)
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10
20
30
40
50
0
10
20
30
● O_2
▲ N_2O

molecular weight.

In our past work with S. faecalis, we had found that the inert gas xenon greatly potentiated the toxic action of oxygen. Why do we now obtain such different results? The most likely explanation for the change is a loss of capacity of S. faecalis to metabolize oxygen when growing in tryptone-glucose-yeast-extract medium. Thus, S. faecalis is now similar to Lactobacillus plantarum. This latter organism has essentially no superoxide dismutase or catalase {McCord et al., 1971} and yet it is oxygen tolerant. The basis for this tolerance is an inability of the bacterium to metabolize oxygen and to produce toxic metabolic products. We had thought of using L. plantarum as a test organism that would allow us to evaluate the narcotic potency of oxygen without the complication of other toxic effects. It appears that S. faecalis is as good a candidate organism and that the experiments we have done will allow us to estimate narcotic potency of oxygen.

However, it should also be pointed out that oxygen must be acting as more than just a narcotic gas against S. faecalis because of the low plate counts obtained when oxygen-poisoned cultures were plated at about 24 hours after inoculation. Cultures that were inhibited to the same degree with nitrous oxide yielded viable counts that were essentially what one would expect from a knowledge of the culture optical density. In all, there seem to be two effects of oxygen - a narcotic action, reflected in slower growth and lower yields, and a lethal action, reflected by low viable counts after long-term exposure. In addition, as indicated above, the response of the organism to oxygen is much more severe when it is growing in phosphate supplemented medium, and presumably, it is able to metabolize oxygen in the presence of high levels of phosphate. Certainly, phosphate-induced oxygen sensitivity is an intriguing phenomenon that bears further investigation.

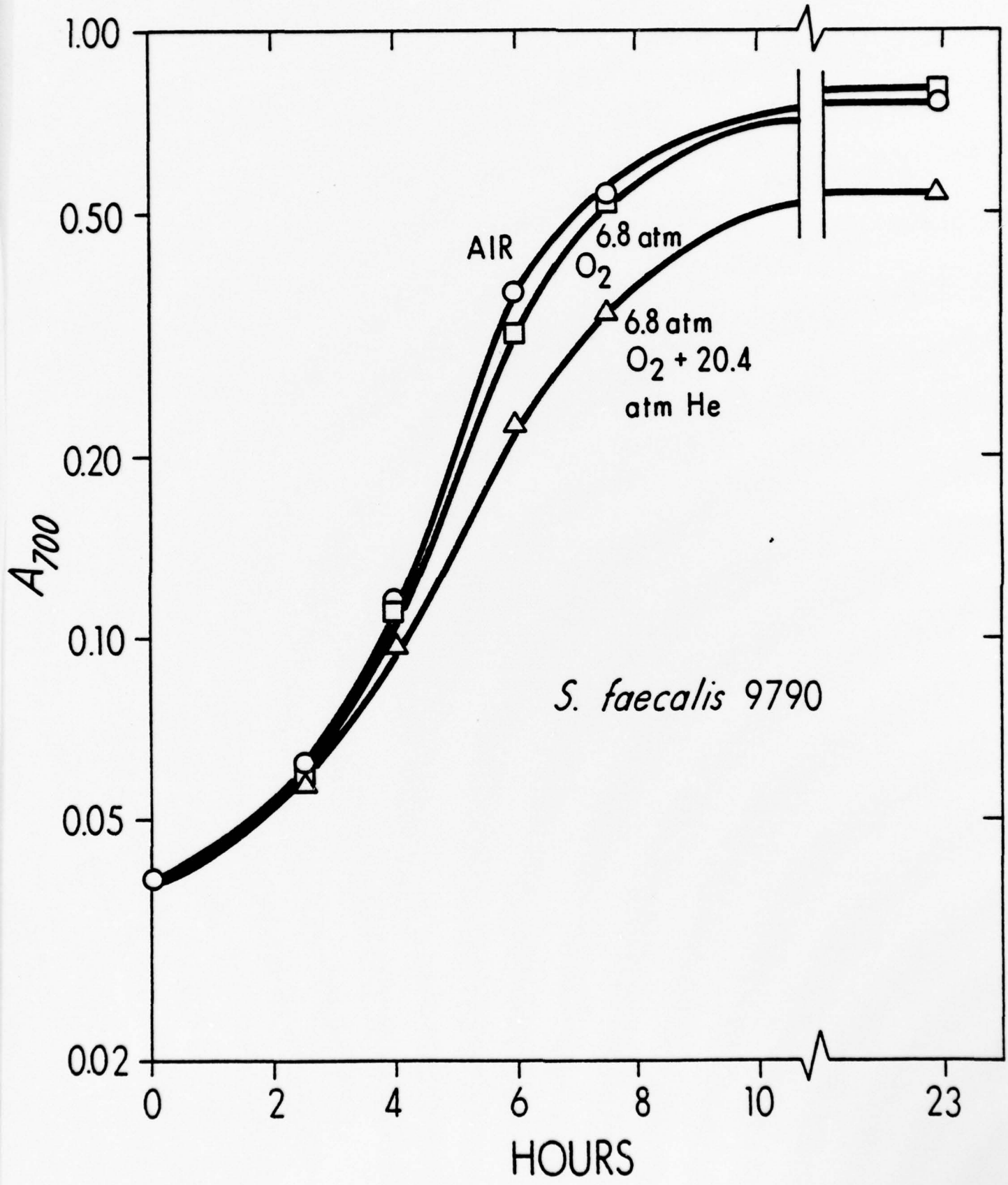
E. Combined actions of oxygen and helium. ZoBell and Hittle {1967} showed that hydrostatic pressure markedly potentiates the toxic action of oxygen for bacterial cells. We initially thought that this potentiation could be due to pressure inhibition of superoxide dismutase, the major protective enzyme of aerobes. However, the results of experiments described in a previous report indicated that superoxide dismutase is remarkably pressure tolerant and is fully active at 1000 atm. We then attempted to repeat the work of ZoBell and Hittle but were unable to do so in a way that we thought was reliable, largely because of the quantities of gas that are required to produce a system saturated with, say, 10 atm of oxygen but without a gas phase. To saturate

10 ml of culture medium with sufficient oxygen to give a concentration of 175 μg/ml - the equivalent of 25 atm O_2 - one would have to start with 7.1 ml of the gas and compress it. The system within the pressure chamber would then have to undergo a contraction of 7.1 ml. It is difficult to accommodate this great a contraction when neoprene rubber stoppers are used with glass vials. The stoppers just cannot move into the vials without developing leaks. The use of plastic syringes for gas experiments has problems also because the plastics have a fair degree of gas permeability. We are currently working on a two-chamber system that makes use of the fluorocarbon FC-80 in which gases are highly soluble. Hopefully, within the next report period, we will have been able to repeat the work of ZoBell and Hittle.

An alternative system for studying the effects of hydrostatic pressure on oxygen toxicity that we explored involves use of a biologically inactive gas. Helium was our first choice, although the data presented above indicates that it is not without specific action on biological systems. However, the relatively small effect that it has on growth of S. faecalis is stimulatory rather than inhibitory. Certainly, it cannot be claimed to have a narcotic effect. The data presented in Fig. 6 indicate that a helium pressure of 20.4 atm does significantly potentiate the toxic action of 6.8 atm O_2, and it seems that ZoBell and Hittle are correct in their views. The slowed growth and reduced yield due to the helium-oxygen combination was accompanied by a major decrease in viability. After 23 hours, the viable count for the culture exposed to 6.8 atm O_2 plus 20.4 atm He was only 30% that of the other two cultures. The overall effect of 20.4 atm He here was to make 6.8 atm O_2 as potent as approximately 16 atm O_2 in the absence of helium. This evaluation of the potentiation may be an underestimate since, as shown in Fig. 2, helium alone significantly enhances growth rate and slightly enhances growth yield.

F. Combined actions of nitrous oxide and helium. The data presented in Fig. 7 show that 20.4 atm nitrous oxide is more potent in its action on S. faecalis in the presence of 20.4 atm helium. The effect is not as great as that seen with oxygen, and even in the presence of helium, nitrous oxide did not appear to kill the organism, at least not over a 24-hour period. It appears that helium enhances rather than reduces the action of nitrous oxide. One might expect the opposite result since helium alone is stimulatory for growth. Investigations are currently underway to find out if this enhancing effect of helium is due to increased hydrostatic pressure.

Fig. 6. Potentiation of the inhibitory effect of oxygen on growth of Streptococcus faecalis by helium. The general experimental procedure is described in the legend to Fig. 1.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6.8 atm O_2 + 20.4 atm He
S. faecalis 9790
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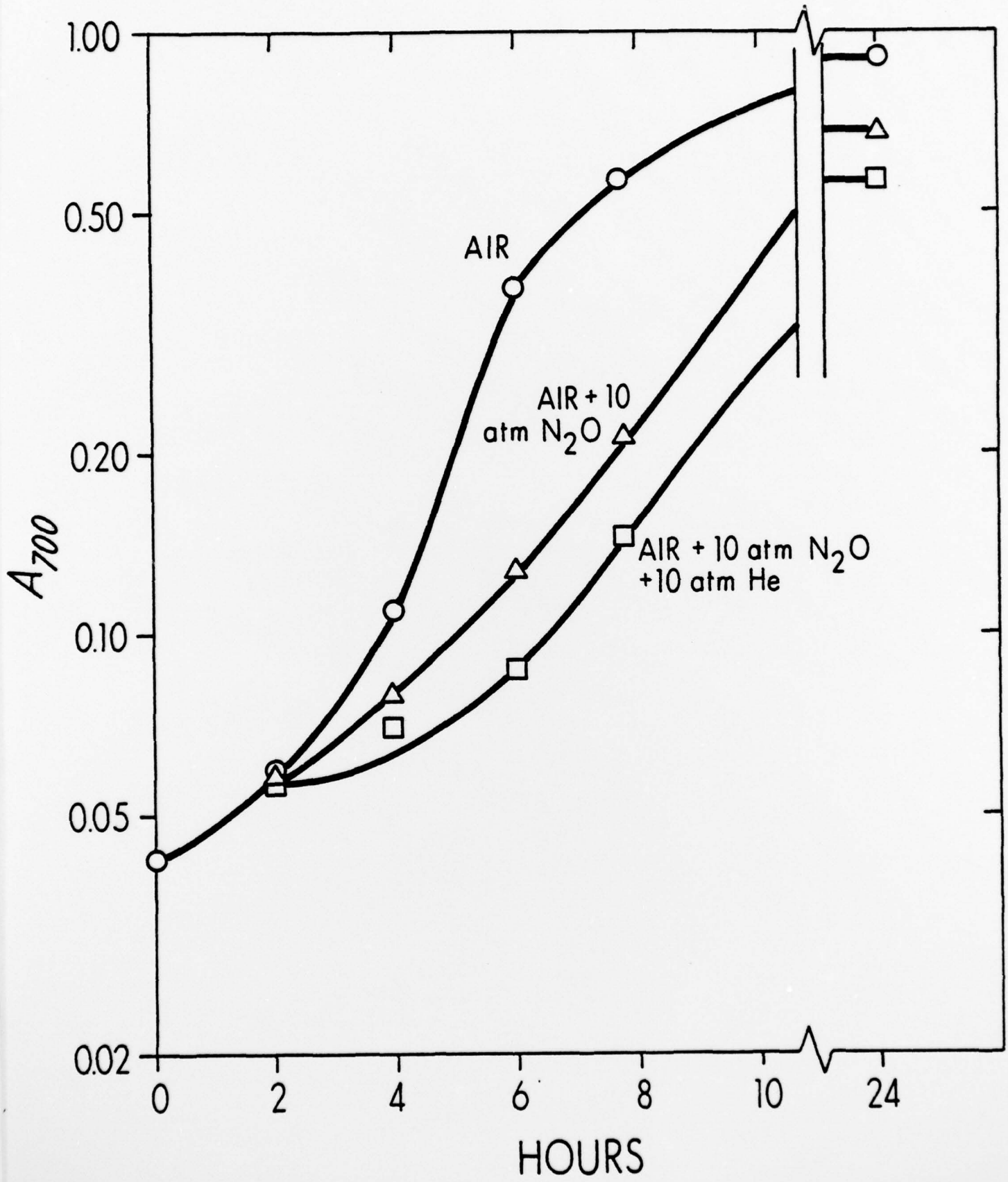
Fig. 7. Combined actions of nitrous oxide and helium on streptococcal growth. The general experimental procedure is described in the legend to Fig. 1.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AIR + 10 atm N_2O
AIR + 10 atm N_2O +10 atm He
0
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24
HOURS

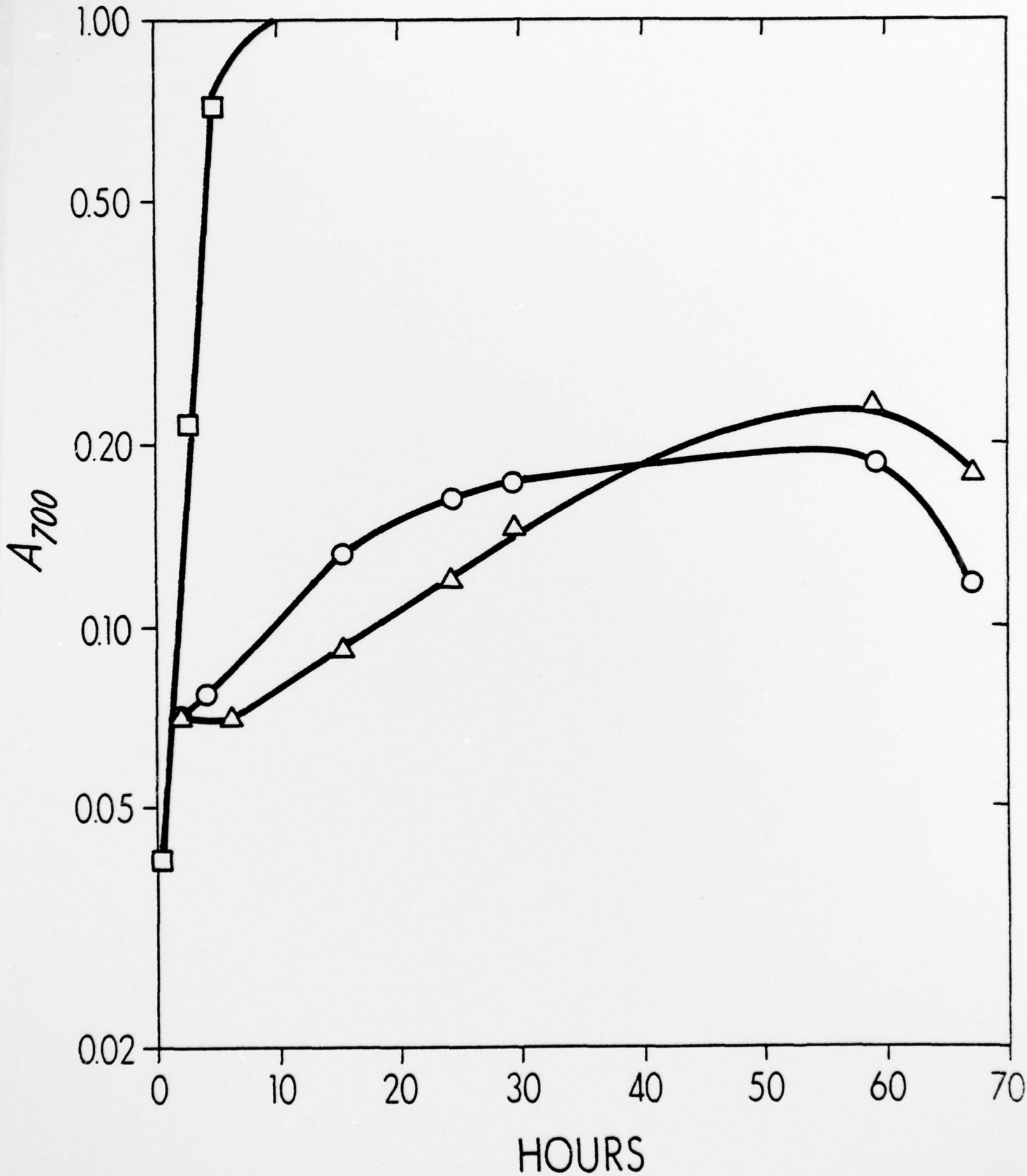
III. Streptococcal Growth at Abyssal-hadal Pressures.

For convenience in our previous experiments with S. faecalis, we had grown the organism at temperatures of 25 to 30°C. The maximum pressure at which growth would occur at these temperatures was 750 atm - in a complex medium with glucose as fuel source and with supplemental $MgCl_2$ or $CaCl_2$. Divalent cations were required for growth at this high pressure and they were found to act by enhancing ATP pooling, possibly through an inhibition of the membrane ATPase of the bacterium. We made a number of attempts to extend the pressure range for growth, including genetic manipulations, but were unsuccessful. However, during studies of pressure-temperature interactions affecting growth, we found that an increase in growth temperature to 37°C rendered S. faecalis significantly more barotolerant, so much so that it could grow at a pressure as great as 900 atm. Sample growth curves are presented in Fig. 8. Apparent activation volumes for growth were 80.9 and 92.4 ml per ml for cultures in ordinary tryptone-glucose-yeast-extract broth and in the same medium supplemented with 50 mM $CaCl_2$. These values are very large, approximately as large as those obtained for S. faecalis growing in lactose medium at 30°C and 400 atm pressure. However, the remarkable finding is that the bacterium will grow at all at such high pressure.

The response of the cells to these high pressures at 37°C is qualitatively different from that to lower pressures at 25 to 30°C. For one thing, divalent cations do not appear to enhance barotolerance at 37°C, and incorporation of 50 mM $MgCl_2$ or $CaCl_2$ into growth media actually slowed growth under pressure. In addition, the cells grown at 37°C and 900 atm were abnormally large, and it appeared that cell division was more severely inhibited than was cell growth. This selective inhibition was never seen with cultures grown at 25 to 30°C. The enlarged bacteria appeared to have normal cell shape, and there was no evidence of lysis in the cultures. In effect, the response of S. faecalis to pressure at 37°C and 900 atm is similar to the response of E. coli to pressures of 300 to 550 atm at temperatures from 25 to 40°C.

This new finding emphasizes our previous view that the barotolerance of bacteria can be greatly altered by changes in growth conditions. S. faecalis is truly remarkable in this respect. With pyruvate as fuel source at 25 to 30°C, growth is completely stopped by a pressure of only about 200 atm. With glucose as fuel at 37°C, it is possible for this same bacterium to grow at 900 atm, close to the upper limit of pressure in the biosphere. In other words, S. faecalis appears to be one of the most barotolerant bacteria known and is in a class with organisms such as Pseudomonas bathycetes.

Fig. 8. Growth of Streptococcus faecalis at 37°C and 900 atm. The growth medium was tryptone-glucose-yeast-extract medium {○} or the same medium supplemented with 50 mM $CaCl_2$ {△}. A curve for growth of the organism in unsupplemented medium at one atm {□} is included for comparison.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0.05
0.02
A_{700}
0
10
20
30
40
50
60
70
HOURS

IV. Low-pressure Effects

A. Enhanced growth at high temperatures. In Fig. 9 some of the data is presented that led to the conclusion that the optimum pressure for bacterial growth is not one atm, at least under many conditions of culture. Here, growth yield for E. coli B cultures in trypticase-soy broth with 0.1% KNO_3 at one, 100 and 200 atm is plotted against growth temperature. Similar results were obtained for growth rate. It can be seen that over a fairly wide range of temperature, from about 25 to nearly 50°C, growth at 100 or 200 atm is greater than that at one atm. Moreover, maximum yields at 100 and 200 atm and 45°C are greater than those at any temperature at one atm. In other words, with this system it is possible to obtain a true enhancement of growth with relatively low pressures. It is apparent also from the figure that below about 20°C the organism becomes highly barosensitive and can be markedly inhibited in its growth by 100 atm.

B. Antibiotic production under pressure. This aspect of the project is still in the preliminary stages of work. However, during the past year, we have made progress in developing a test system. Most antibiotics are produced by aerobic organisms, and optimal production requires culture aeration, which is difficult under pressure with the equipment we now have. One antibiotic that is produced by a facultative organism is nisin. The producer, Streptococcus lactis, can grow in air but does not use oxygen. The antibiotic is relatively widely used for preservation in the food industry, particularly for dairy products, and it is active against a range of gram-positive and gram-negative bacteria. We have used a biological assay for the antibiotic with Bacillus megaterium KM as an indicator of activity. Molten agar at a temperature of 45 to 50°C is seeded with B. megaterium, and standard pour-plates are prepared. After the agar has hardened, uniform wells are cut in it and are filled with cell extracts that are thought to contain nisin. The plates are incubated at 30°C for 48 hours before zones of inhibition are measured. Nisin is heat stable but alkaline sensitive. Therefore, samples can be heated to 63°C to inactivate antibacterial factors in culture extracts. It is possible also to destroy nisin by raising the pH to 11, and after subsequent back titration with acid, samples exposed to pH 11 for 30 minutes are devoid of antibacterial activity.

Our preliminary results show that nisin is produced are pressures of 100 or 200 atm and that production per cell is somewhat enhanced under pressure. However, we have had to contend with unusual heat and pressure sensitivity of S. lactis. Growth at temperatures above 37°C and 100 or 200 atm is poor. Clearly, more experiments are needed. It is possible that we may not be able to enhance nisin production by use of hydrostatic pressure. However, this result will not mean that it will not be possible to enhance production of other microbial metabolites. Pressure-temperature

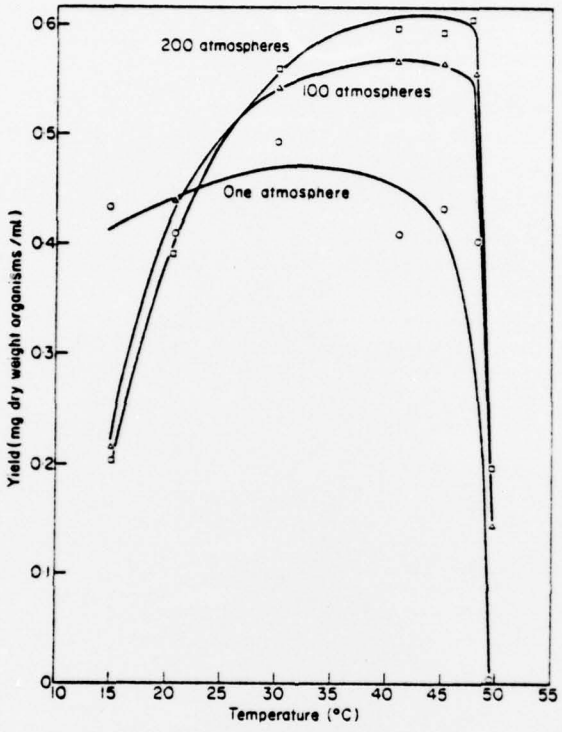

Fig. 9. Growth yields of Escherichia coli cultures, grown at one atm {○}, 100 atm {△} and 200 atm {□}, in relation to the growth temperature. The medium used was trypticase-soy broth containing 0.1% {w/v} KNO_3.

responses are highly specific and depend on ΔH and ΔV values for the particular process under study. Therefore, we shall be studying a number of processes in addition to nisin production.

C. Enhanced barosensitivity at low temperature. The data presented in Fig. 9 show also that E. coli becomes highly pressure sensitive at low temperature. It is possible to markedly inhibit growth with a pressure as low as 100 atm. In other experiments with cultures growing at 9°C, we found that it is possible to cause major inhibition with only 50 atm. This sort of data has been used by us to develop the view that low pressures are of major ecological importance in the continental-shelf and continental-slope regions of the ocean, in the shallow layers of the open ocean and in fresh water. Most organisms growing in natural environments are not growing under optimal conditions, and presumably, they are highly barosensitive.

During the past year, we have attempted to extend our view to a molecular level by showing that enzymes can become extremely barosensitive when environmental conditions are not optimal. Unfortunately, the first enzyme we choose for study, lactic dehydrogenase, has an activity that can be characterized by a positive ΔH and a positive ΔV. It is stimulated by temperature increase from 11 to 22°C but inhibited by increased pressure up to 1000 atm. However, it is more barotolerant at the lower temperature. At 22°C, activity is completely stopped by 1000 atm, while at 11°C, 1000 atm causes only about 40% decrease in activity. For these experiments, the enzyme was assayed by following the change in absorbance at 340 nm due to reduction of NAD^+. The assay was set up so that the enzyme was rate limiting. This sort of behavior can be expected from one class of enzymes. It very clearly demonstrates the view we had proposed previously that the simple gas laws are not applicable to condensed biological systems. In other words, increased pressure and increased temperature are not always antagonistic. It depends on the types of chemical bonds that are undergoing change in any particular situation. The background for this thesis is presented in a recent review article {Marquis, 1976}.

During the next support period, we shall be investigating other enzymes to obtain examples of the class which displays increased barosensitivity when the temperature is lowered. This class should be the major class of enzymes in a classification based on effects of pressure and temperature.

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Investigations during the past year of the effects of high-pressure narcotic gases have confirmed previous reports of inhibition of microbial growth and differentiation. Spore germination was found to be somewhat more sensitive than was growth, and for example, germination of Bacillus cereus spores could be completely suppressed by only 10 atmospheres of nitrous oxide, compared with some 25 atmospheres required for 50% inhibition of growth of Streptococcus faecalis. High-pressure helium at 24 to 41 atmospheres was found to act oppositely in that it stimulated streptococcal growth and

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replication. This stimulation could be related to the finding that helium has negative narcotic potential for animals. Helium did act to enhance the narcotic potential of nitrous oxide and to increase oxygen toxicity, possibly because of a hydrostatic pressure effect rather than a specific gas effect. Oxygen appeared to act essentially as a narcotic gas in its inhibition of S. faecalis growth in a medium prepared with tryptone, glucose and yeast extract. The oxygen pressure required for 50% growth inhibition was about 25 atmospheres, approximately the pressure of nitrous oxide required for the same effect. However, long-term exposure to oxygen resulted in cell death, while similar exposure to nitrous oxide resulted in no killing. Moreover, supplementation of the complex growth medium, or of a defined one, with phosphate buffer resulted in markedly enhanced oxygen sensitivity of the bacterium, and it was possible to obtain 50% growth inhibition with only about 15 atmospheres of oxygen.

Further studies of pressure-temperature interactions affecting microbial growth and physiology were undertaken. Efforts were made to enhance metabolite production by bacteria with increased hydrostatic pressure and to study molecular aspects of the interaction.

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